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GIT2 deficiency attenuates concanavalin A-induced hepatitis in mice

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ABSTRACT

G protein-coupled receptor kinase interactor 2 (GIT2) is a signaling scaffold protein involved in regulation of cytoskeletal dynamics and the internalization of G protein-coupled receptors (GPCRs). The short-splice form of GIT2 is expressed in peripheral T cells and thymocytes. However, the functions of GIT2 in T cells have not yet been determined. We show that treatment with Con A in a model of polyclonal T-lymphocyte activation resulted in marked inhibitions in the intrahepatic infiltration of inflammatory cells, cytokine response and acute liver failure in *Git2*$^{-/-}$ mice. CD4^{+} T cells from *Git2*$^{-/-}$ mice showed significant impairment in proliferation, cytokine production and signal transduction upon TCR-stimulated activation. Our results suggested that GIT2 plays an important role in T-cell function *in vivo* and *in vitro*.

1. Introduction

Immune cells, including lymphocytes and Kupffer cells, constitute approximately 45% of the total non-hepatocyte cells in the normal liver [1,2]. Nearly all of the innate immune cells in the liver have been reported to be involved in the diverse liver injuries observed in experimental animal models and/or human clinical investigations [1]. It is well established that Con A induced liver damage in mice is a typical model that closely resembles several pathological properties of human viral or autoimmune hepatitis [3]. Activated T cells play a critical role in Con A-induced liver damage [4]. Tiegs found that pretreatment of mice with monoclonal anti-CD4 antibody inhibited Con A-induced hepatitis, suggesting that CD4^{+} T lymphocytes are required for the initiation of the immunological response [4]. In addition, immune cells, including CD8^{+} T cells, natural killer (NK) cells, natural killer T cells, Kupffer cells/macrophages, neutrophils and eosinophils, have also been reported to be involved in Con A-induced hepatitis by either cell-to-cell contact or the secretion of pro-inflammatory cytokines or reactive oxygen species [3].

G protein-coupled receptor kinase interacting protein 2 (GIT2) is a signaling scaffold protein involved in the regulation of cytoskeletal dynamics, GPCR internalization and membrane trafficking [5]. GIT2 has numerous splice variants, and the longest splice form of GIT2 (GIT2-long) is expressed in a wider range of cells, whereas GIT2-short is specific to immune cells [6]. GIT2 is a ubiquitous multi-domain protein that has an N-terminal ARF-GAP domain, three Ank repeats, a Spa2-homology domain (SHD), a coiled-coil (CC) domain and a paxillin-binding site (PBS) [5]. The GIT2-short lacks the CC domain and PBS [5]. GIT2 regulates diverse cellular functions through interacting with various proteins. For example, GIT2 agonist GPCR endocytosis through regulating ARFs via ARF-GPA domain and the phosphorylation of GIT2 at PBS via Src and FAK is required for its recruitment to focal complexes, which is essential for the formation of normal lamellipodia during cell spreading [6,7]. In addition, the main binding partner for the SHD of GIT2 is PIX, through which GIT2 can regulate cytoskeletal dynamics via interacting with PAK1 and the small GTPases Rac1 and Cdc42 [8]. GIT/PIX complexes are known to function as scaffolds for a variety of signaling proteins, including GRKs, PAKs, FAK, the MEK1-ERK1/2 mitogen-activated protein kinases, and phospholipase C [5]. Previous studies have shown that GIT

Abbreviations: GIT2, G protein-coupled receptor kinase interactor 2; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; TCR, T cell receptor; Con A, concanavalin A; PMA, 4b-phorbol 12-myristate 13-acetate

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interacts constitutively in a tri-molecular complex with PIX and PAK in T cells and that this complex is recruited to the T cell immunological synapse by PIX [9,10]. The mutation of PIX in mice results in neutrophil that are defective in orienting and migrating toward a chemoattractant, and this phenotype resembles that of GIT2-knockout mice [11–13]. Moreover, PIX-knockout T lymphocytes showed defective TCR-induced proliferation and signaling but enhanced basal migration [12]. Interestingly, *Git2*$^{-/-}$ double-positive (DP) thymocytes display increased migration toward SDF-1 and CCL25 *in vitro*, suggesting that GIT2 plays a key role in regulating the chemokine-mediated motility of DP thymocytes [14]. In addition, through analyzing a genome-wide association study (GWAS) dataset, significant association between the single nucleotide polymorphisms (SNP) in GIT2 and abnormal values of HDL cholesterol has been reported [15]. Clinically, in addition to metabolic syndrome, it is generally thought that an abnormal value of HDL-C results from liver dysfunction [16], which suggests a potential role of GIT2 in liver disease. Taken together, these data show that GIT2 may play an important role in the regulation of T cell function and in liver disease.

In this study, we utilized *Git2*$^{-/-}$ mice to investigate the role of GIT2 in immune cells during T cell-mediated hepatitis. We found that GIT2 deficiency in mice led to a spontaneous reduction of basal CD4$^+$ T cells and NKT cells in the liver. Treatment with Con A in a model of polyclonal T lymphocyte activation resulted in marked inhibitions in the intrahepatic infiltration of inflammatory cells, cytokine response and acute liver failure in *Git2*$^{-/-}$ mice. CD4$^+$ T cells from *Git2*$^{-/-}$ mice showed significant impairments in proliferation, cytokine production and signal transduction upon TCR-stimulated activation. Our data demonstrate that GIT2 plays an important role in Con A-induced hepatitis and may therefore be a potential target for therapeutic intervention in acute liver diseases.

2. Results

2.1. GIT2 depletion attenuated Con A-induced immunological hepatic injuries

Heterozygous GIT2-knockout mice were bred to produce *Git2*$^{+/+}$ controls and homozygous GIT2-knockout (*Git2*$^{-/-}$) mice. Positive founder mice were identified by RT-PCR and Western bolt analyses (Fig. 1A and B). GIT2 was completely absent from the total thymocytes, splenic CD4$^+$ T cells and liver tissues, as determined using an antibody against the mouse GIT2. In agreement with previous reports [13], adult *Git2*$^{-/-}$ mice showed no gross phenotypic abnormalities but often developed splenomegaly (Fig. 1C and D) and an increased CD4$^+$/CD8$^+$ ratio in the liver MNCs (Table 1). To examine the effects of GIT2 on immune-mediated hepatitis, we applied the Con A-induced hepatitis model in six- to eight-week-old male *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice. We observed that the serum AST and ALT levels after the injection of 15 mg/kg Con A were significantly lower in *Git2*$^{-/-}$ mice compared with *Git2*$^{+/+}$ mice (Fig. 1E). Histological analyses of liver tissue sections obtained 24 h after the administration of 15 mg/kg Con A indicated that *Git2*$^{-/-}$ mice were less sensitive to Con A-induced hepatic injury (Fig. 1F). Liver tissue sections of *Git2*$^{-/-}$ mice showed several solitary areas of necrotic tissue characterized by standard morphologic criteria (Fig. 1F – d–f), and the majority of hepatocytes were not affected. However, liver tissue sections of *Git2*$^{+/+}$ mice showed widespread areas of necrosis (Fig. 1F – a) with extensive infiltration of mononuclear cells within the liver lobules (Fig. 1F – b) and around the central veins and portal tracts (Fig. 1F – c), indicating an ongoing inflammatory process. Consistent with these findings, the percentage of liver tissue with necrotic damage was markedly lower in *Git2*$^{-/-}$ mice (Fig. 1G). The livers of *Git2*$^{-/-}$ mice also demonstrated a significant decrease in hepatocyte apoptosis 24 h after treatment with 15 mg/kg Con A (Fig. 1H): the liver of *Git2*$^{+/+}$ mice showed 37% hepatocyte apoptosis, whereas only 13% apoptosis was observed in the *Git2*$^{-/-}$ mice. Taken together, these data indicated that mice deficient in GIT2 appeared highly resistant to liver injuries induced by Con A.

To test whether GIT2 depletion also protects mice against chemically induced liver injury, *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice were injected i.p. with CCl_4. The results showed no significant differences in liver injury between *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice (Fig. 2A and B), which may be due to a different mechanism of liver injury in this model, indicating the potential role of GIT2 in T cell-mediated liver injury.

2.2. GIT2 depletion suppressed lymphoid cells infiltration to liver after Con A treatment

To examine whether GIT2 depletion affects the influx of MNCs in the liver after Con A injection, we first analyzed the steady-state composition of immune cells in the liver of *Git2*$^{-/-}$ mice. The liver MNCs were isolated and subjected to flow cytometry analysis. As shown in Fig. 3A, the total number of mononuclear cells in the liver of *Git2*$^{-/-}$ mice was equal to that in *Git2*$^{+/+}$ mice. However, the numbers of basal CD4$^+$ T cells and NKT cells were significantly reduced in the liver of *Git2*$^{-/-}$ mice, whereas the CD8$^+$ cells were increased compared with the *Git2*$^{+/+}$ controls. Other liver MNC subsets, such as NK cells and B cells, were not influenced (Fig. 3A).

After Con A injection, although increased, the number of MNCs in the liver of *Git2*$^{-/-}$ mice was significantly lower than that found in *Git2*$^{+/+}$ mice (Fig. 3A). Moreover, the degree of increase was much smaller in the number of *Git2*$^{-/-}$ liver MNC subsets, including CD4$^+$, CD8$^+$ T cells and CD19$^+$ B cells (Fig. 3A). For example, the absolute number of CD4$^+$ T cells in the liver of *Git2*$^{+/+}$ mice increased approximately two-fold but only increased 20% in GIT2$^{-/-}$ mice. Consistent with the previous report [17], NKT cells were markedly decreased in both *Git2*$^{-/-}$ and *Git2*$^{+/+}$ mice. Additionally, there was no significant difference in the number of NK cells between *Git2*$^{+/+}$ and *Git2*$^{-/-}$ mice. We also analyzed the percentage of these cell types and the pattern is similar with their number (Table 2). Although the absolute number was the same with control, the percentage of T regulatory cells (Tregs; CD4$^+$CD25$^+$Foxp3$^+$) in the liver of *Git2*$^{-/-}$ mice was significantly higher than that in *Git2*$^{+/+}$ mice after Con A injection (Fig. 3B). In addition, we assessed the absolute number and the percentage of Tregs in splenocyte. As shown in Fig. 3C, the number of splenic mononuclear cells in *Git2*$^{-/-}$ mice was more than 2-fold higher than that of *Git2*$^{+/+}$ mice, which consistent with previous reporter [13]. Prior to Con A treatment, no statistically significant difference was detected in the populations of CD4$^+$ and Tregs in spleens of *Git2*$^{-/-}$ and *Git2*$^{+/+}$ mice. After Con A treatment, the absolute number and percentage of Tregs were significantly higher in *Git2*$^{-/-}$ spleen than *Git2*$^{+/+}$. We also detected the apoptosis of liver MNCs and splenocyte and absence of GIT2 did not significantly change the Con A-induced apoptosis (Fig. 3D). These data suggest that GIT2 deficiency attenuate Con A-induced infiltration of inflammatory cells in the liver which protect *Git2*$^{-/-}$ mice from liver injury.

2.3. Lower levels of proinflammatory cytokines in Git2$^{-/-}$ mice after Con A injection

Proinflammatory cytokine production is a key component of Con A-induced liver injury. We therefore assessed the levels of TNF-α, INF-γ, IL-2, IL-4, IL-6, IL-17A, and IL-10 in the mice serum.